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Neurotrophin Binding to the p75 Receptor Modulates Rho Activity and Axonal Outgrowth

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Summary

While the neurotrophin receptor p75^{NTR} is expressed by many developing neurons, its function in cells escaping elimination by programmed cell death remains unclear. The lack of intrinsic enzymatic activity of p75^{NTR} prompted a search for protein interactors expressed in the developing retina, which resulted in the identification of the GTPase RhoA. In transfected cells, p75^{NTR} activated RhoA, and neurotrophin binding abolished RhoA activation. In cultured neurons, inactivation of Rho proteins mimicked the effect of neurotrophins by increasing the rate of neurite elongation. In vivo, axonal outgrowth was retarded in mice carrying a mutation in the *p75^{NTR}* gene. These results indicate that p75^{NTR} modulates in a ligand-dependent fashion the activity of intracellular proteins known to regulate actin assembly.

Introduction

The neurotrophin receptor p75 (p75^{NTR}) is a membrane glycoprotein that binds all neurotrophins identified so far (reviewed by Bothwell, 1995). In addition to its ability to activate sphingomyelin hydrolysis (Dobrowsky et al., 1994) and NF-κB (Carter et al., 1996), p75^{NTR} has also been linked to programmed cell death (Rabizadeh et al., 1993). Neurotrophin-induced, p75^{NTR}-mediated cell death has been described with primary cultures of oligodendrocytes (Casaccia-Bonnefil et al., 1996) and of neurons (Bamji et al., 1998; Davey and Davies, 1998), as well as in vivo (Frade et al., 1996; Bamji et al., 1998; Frade and Barde, 1999). However, the expression of p75^{NTR} by many nondying neurons suggests that this receptor is likely to be involved in other biological functions during development. A major difficulty in clarifying the biological role of this receptor has been the nonenzymatic nature of its cytoplasmic domain. Indeed, like all other members of this large gene family, including FAS/Apo-1/CD95 and both tumor necrosis factor receptors (Nagata, 1997), p75^{NTR} needs to associate with cytoplasmic proteins to exert its signaling functions.

p75^{NTR} has an intriguing pattern of expression in the developing nervous system, including in particular major populations of neurons projecting axons over long distances (Buck et al., 1987; Ernfors et al., 1988; Yan and Johnson, 1988; Large et al., 1989; von Bartheld et al., 1991). Motor neurons in the spinal cord, most sympathetic and sensory neurons in the peripheral nervous system, as well as cerebellar Purkinje cells and retinal ganglion cells all express p75^{NTR} at high levels during the outgrowth of axons. In dendrite-bearing neurons, p75^{NTR} is also expressed during the time of dendritic arborization. Typically, the expression of p75^{NTR} is markedly downregulated following the arrival of axon terminals in their target tissues (Yan and Johnson, 1988). Some neurons markedly upregulate p75^{NTR} after section of their axons, including those able to regenerate axons after lesion such as the spinal cord motor neurons (Ernfors et al., 1989). Finally, the most extensively studied population of migrating cells in the nervous system, the neural crest cells, also express p75^{NTR} from the beginning of their segregation from the dorsal neural tube (Stemple and Anderson, 1992).

In order to elucidate the signaling pathway of p75^{NTR} and possible novel biological functions, a yeast two-hybrid screen was carried out using the intracellular domain of p75^{NTR} as a bait. This resulted in the identification of the GTPase RhoA as a p75^{NTR} interactor. The implications of this interaction are examined with regard to the activity of Rho in biochemical and cellular assays.

Results

p75^{NTR} Interacts with RhoA

During early development, p75^{NTR} is expressed at high levels by newly born chick retinal ganglion cells (von Bartheld et al., 1991). While essentially all dying cells express p75^{NTR}, it was also observed that many more cells express p75^{NTR} than die (Frade et al., 1996). This observation led us to prepare an expression library from RNA isolated from E5 chick retinae in the hope of identifying novel p75^{NTR} interactors. The library was screened in a yeast two-hybrid system using the intracellular domain (ICD) of chick p75^{NTR}, fused with a GAL4 DNA-binding domain (Figure 1). Two positive clones were found to be identical and to contain the entire open reading frame of RhoA. Constructs containing only the "linker region" of p75^{NTR} did not interact with RhoA, while those including the six α helices of p75^{NTR} did (Figure 1).

Coimmunoprecipitation studies were then performed using 293 cells cotransfected with the p75^{NTR} ICD and wild-type RhoA tagged with a hemagglutinin epitope (HA-RhoA wt) (Figure 2). In line with the yeast two-hybrid results, HA-Rho efficiently precipitated p75^{NTR}. In an attempt to test if the interaction of Rho depends on its state of activation, we then tested the constitutively active mutant HA-Val^{14}-RhoA. Because Val^{14}-RhoA exhibits reduced GTPase activity and is insensitive to Rho GTPase-activating proteins, it exists mainly in a GTP-bound form (Ridley and Hall, 1992). In contrast to wild-type RhoA, only a faint signal could be detected, in spite of comparable levels of expression of wild-type and mutant proteins in 293 cells (Figure 2). Attempts to pull down full-length p75^{NTR} with HA-RhoA from HN10 cells

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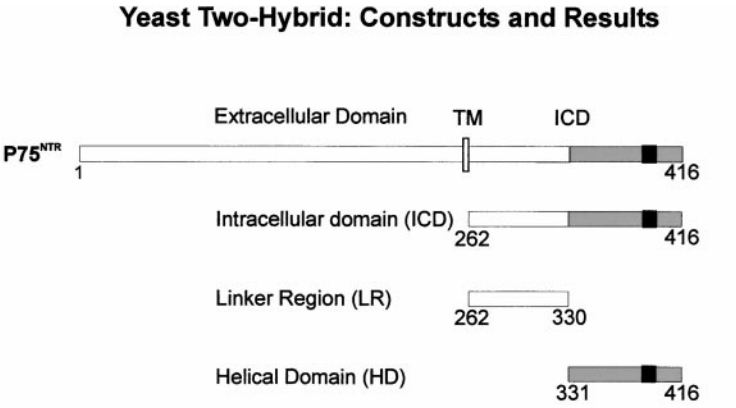

Gal4DBD Plasmid	Gal4AD Plasmid	Growth Assay	β-Galactosidase Colony Assay
pBD-Gal4-P75^{NTR} ICD	pAD-Gal4-RhoA wt	+	+
pBD-Gal4-P75^{NTR} LR	pAD-Gal4-RhoA wt	-	-
pBD-Gal4-P75^{NTR} HD	pAD-Gal4-RhoA wt	+	+
pBD-Gal4-p53	pAD-Gal4-SV40-T	+++	+++

Figure 1. p75^{NTR} Interacts with RhoA in the Yeast Two-Hybrid System

p75^{NTR} ICD, the linker region, or the six α helices–containing domain (see Liepinsh et al., 1997) were expressed as fusion proteins with the GAL4-binding domain (BD). Their interaction with RhoA wt expressed as a fusion protein with the GAL4 activation domain (AD) was examined by both a growth assay and a filter assay for β-galactosidase activity. TM indicates the transmembrane domain. The black rectangle in ICD indicates the approximate position of the mastoparan-like domain.

failed. The higher concentrations of detergents that had to be used to solubilize the receptor presumably disrupted the interaction (data not shown).

p75^{NTR} Constitutively Activates RhoA

The inability of the Val^{14}-RhoA mutant to immunoprecipitate p75^{NTR} suggested that p75^{NTR} is unlikely to be a target of Rho, since it is typically the GTP-bound, active form of Rho that interacts with its effectors. This prompted us to examine whether p75^{NTR} might function instead as a Rho regulator. 293 cells transiently transfected with wild-type HA-RhoA were ^{32}P labeled, and the radioactivity associated with RhoA was determined by immunoprecipitation of RhoA followed by thin-layer chromatography. As Rho proteins have a high intrinsic GTPase activity, this assay precludes the detection of radiolabeled GTP and reflects the nucleotide exchange rates of Rho proteins (Laudanna et al., 1996; Crespo et al., 1997). We compared the levels of [^{32}P]GDP associated with RhoA in the presence or absence of cotransfected p75^{NTR} ICD or of empty vector. While the levels were low in the absence of p75^{NTR}, a massive increase was observed in the presence of p75^{NTR} (Figure 3A). The extent of the increase was dependent on the expression levels of p75^{NTR}. Similar results were obtained when RhoB (Figure 3A) or RhoC (data not shown) were used instead of RhoA.

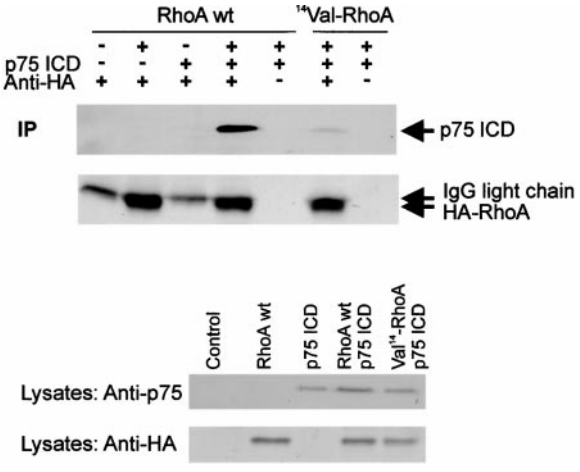

Figure 2. Wild-Type Rho, but Not the Active Form of Rho, Precipitates p75^{NTR} ICD

(Top) Coimmunoprecipitation of p75^{NTR} ICD with RhoA. HA-RhoA wt or HA-Val^{14}-RhoA was immunoprecipitated with hemagglutinin antibodies from cells transfected with RhoA and/or p75^{NTR} ICD. The blot was reacted with antibodies to p75 ICD.

(Bottom) Expression of tagged Rho (wild type and mutant) or p75^{NTR} in cell lysates.

Ligand Binding to p75^{NTR} Decreases Nucleotide Labeling of Rho

Not only the cytoplasmic domain of p75^{NTR} but also the full-length receptor markedly increases [^{32}P]GDP-RhoA levels (Figure 3B). This opened the possibility of testing the modulation of [^{32}P]GDP-Rho levels by the ligands of p75^{NTR}. When cells were incubated with nerve growth factor (NGF, 50 ng/ml) during the ^{32}P labeling period (16 hr), guanyl nucleotide labeling was greatly reduced (Figure 3B). We then tested two other p75^{NTR} ligands, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Both were found to also inhibit nucleotide labeling of RhoA by p75^{NTR}, though to a lesser extent (Figure 3B). As NGF had no effect upon cells transfected with the cytoplasmic domain of p75^{NTR} (data not shown), we conclude that the reduction of nucleotide labeling of RhoA by NGF is a consequence of its binding to the extracellular domain of p75^{NTR}. We next used HN10 cells,

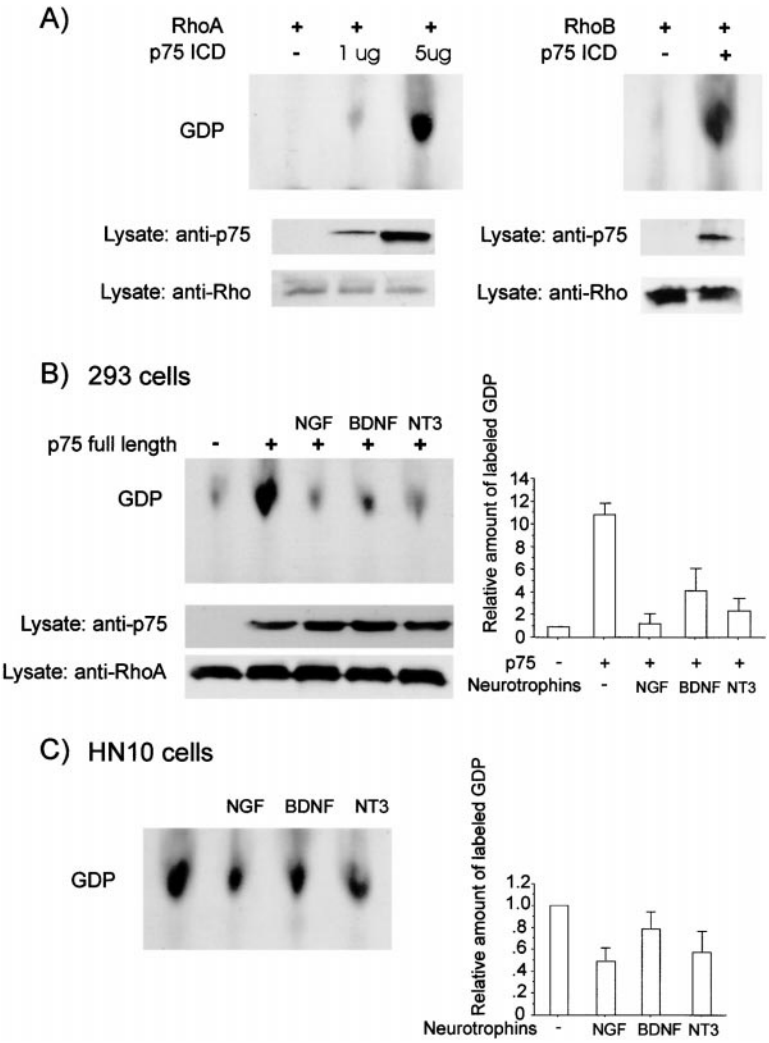

Figure 3. Precipitation of [32P]GDP-RhoA or RhoB

(A) Following 32P labeling, cells transfected with tagged RhoA or RhoB and with or without p75NTR ICD were immunoprecipitated with the anti-HA antibody. The radioactivity precipitated with Rho comigrated with a GDP standard. More GDP is coprecipitated when 5 μg p75 ICD is used, compared with 1 μg; 5 μg p75 ICD was used in the RhoB experiment.

(B) 293 cells were transfected with full-length p75NTR and tagged RhoA and incubated with or without neurotrophins (50 ng/ml each) during the 32P labeling period. [32P]GDP was quantified by scraping the thin-layer plates and counting. Values represent relative amount of radioactivity and are expressed as means ± SEM of three experiments.

(C) HN10 cells were transfected with tagged RhoA and incubated with or without neurotrophins (50 ng/ml each) during the 32P labeling period. [32P]GDP was quantified as in (B).

a cell line that constitutively expresses p75NTR (Lee et al., 1990). Cross-linking experiments with radiolabeled NGF indicated that p75NTR was the only cell surface protein binding NGF in these cells (data not shown). While the extent of the decrease of Rho labeling following neurotrophin addition was less than that seen with 293 cells, a clear and significant reduction was observed with NGF (Figure 3C).

NGF Causes a Rapid Loss of RhoA Activation

To examine the time course of the action of neurotrophins on Rho inactivation, we took advantage of a recently developed assay allowing the precipitation of GTP-Rho using the Rho-binding domain (RBD) of the effector protein Rhotekin (Ren et al., 1999). RBD only interacts with GTP-bound Rho. It inhibits both the intrinsic GTPase activity of Rho and also its GTPase activity enhanced by GTPase-activating proteins. Thus, RBD can be used as a convenient method to affinity precipitate cellular GTP-Rho (Ren et al., 1999). The total amount of GTP-Rho at a given time point can thus be measured.

This assay revealed that within 10 min following the addition of NGF (50 ng/ml), extracts of 293 cells transfected with full-length p75NTR and RhoA contained dramatically less GTP-RhoA (Figure 4). This inactivation persisted for several hours following continuous exposure to NGF (Figure 4). Similar results were obtained with BDNF and NT-3 (also used at 50 ng/ml).

p75NTR-Mediated Modulation of Neurite Outgrowth

To investigate the significance of Rho regulation by p75NTR in neurons, we used ciliary neurons isolated from chick embryos. Not only do these cells express p75NTR and not the NGF tyrosine kinase receptor TrkA (see Figure 5A), but they also have long been known to respond to NGF by an increase in the rate of neurite elongation (Collins and Dawson, 1983). Antibodies to RhoA and to p75NTR revealed that all cells express these proteins (Figure 5B). While ciliary neurons, like most other embryonic neurons, spontaneously extend neurites on a polyornithine-laminin substrate, the addition of NGF measurably increases neurite length, compared with untreated control cultures (Figures 5C and 6). Similar effects were observed following the addition of BDNF or

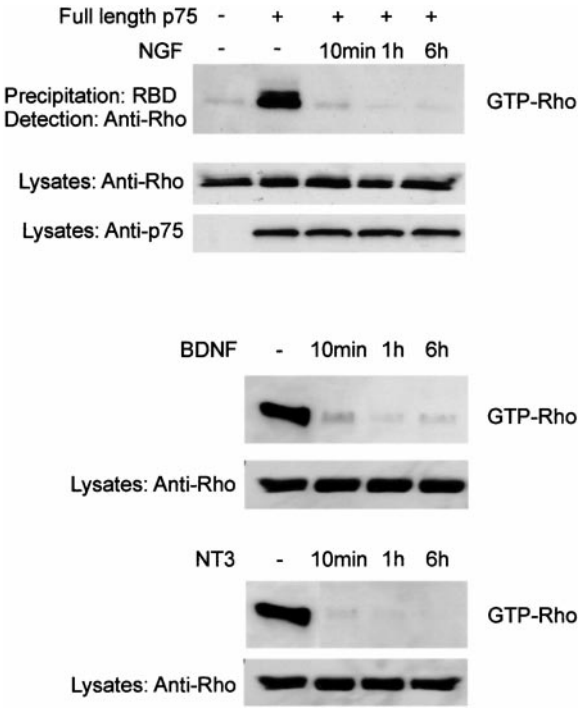

Figure 4. Affinity Precipitation of GTP-Rho Using the Rho Effector Protein Rhotekin

While the levels of GTP-Rho were massively increased by coexpression of full-length $p75^{NTR}$ with RhoA, background levels were observed already 10 min after the addition of NGF to the medium. Similar results were obtained with BDNF and NT-3. Neurotrophins were used at the concentration of 50 ng/ml.

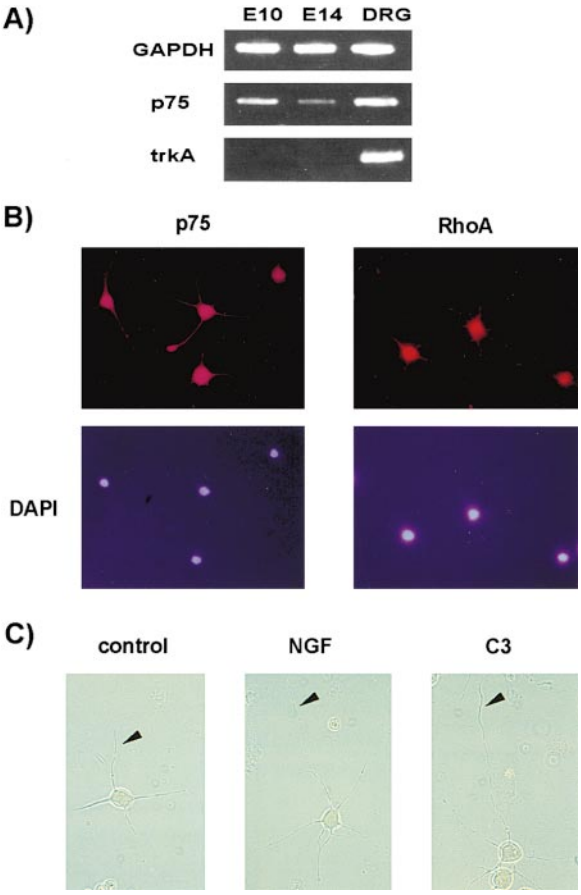

Figure 5. $p75^{NTR}$-Mediated Neurite Outgrowth of Ciliary Neurons

(A) RT–PCR analysis of RNA extracted from ciliary ganglia. While the *trkA* gene is not expressed in these ganglia at E10 or at E14, $p75^{NTR}$ can clearly be detected at E10. At E14, the levels are lower, indicating a developmental downregulation of $p75^{NTR}$, as observed in most other tissues.

(B) Immunocytochemistry of $p75^{NTR}$ (left) or RhoA (right) using monoclonal antibodies. Essentially all of the neurons express both proteins in E10 ciliary neurons.

(C) Ciliary neurons as observed 5 hr after plating on a polyornithin-laminin substrate (left, control), in the presence of NGF (50 ng/ml, middle) or after trituration of C3 into the cells (right).

NT-3, albeit to a lesser extent (Figure 6). To demonstrate that neurotrophins stimulate neurite outgrowth by a $p75^{NTR}$-dependent mechanism, we made use of the observation that human NT-4/5 is not active on avian neurons (Davies et al., 1993) and used it as a competitive antagonist. While inactive on its own at 50 μg/ml (Figure 6) or 50 ng/ml (data not shown), NT-4/5 at the concentration of 50 μg/ml completely abolished the effects of NGF at 50 ng/ml (Figure 6).

We then tested the hypothesis that the effects of NGF on neurite outgrowth are caused by a decreased activation of Rho proteins. In order to specifically inactivate the Rho protein, we employed the exoenzyme C3 transferase from *Clostridium botulinum*, which ADP ribosylates Rho proteins (Aktories et al., 1987; Narumiya et al., 1988). The recombinant C3 transferase was introduced into the cytoplasm of ciliary neurons by trituration (see Borasio et al., 1989). Like NGF, the C3 transferase caused neurites to elongate faster (Figure 6).

Finally, we tested the prediction that Rho activation is located downstream of $p75^{NTR}$ in the signaling pathway activated by NGF. When introduced into ciliary neurons, the constitutively active mutant Val^{14}-RhoA protein completely inhibited the action of NGF on neurite outgrowth (Figure 6). On its own, the mutant Val^{14}-RhoA had no effect on neurite outgrowth, suggesting that the levels of $p75^{NTR}$ expressed in the ciliary neurons saturate Rho-dependent effects on inhibition of neurite outgrowth.

Reduced Outgrowth of Spinal and Sensory Nerves in *p75^{NTR}*-Deficient Mice

Prompted by the observed influence of neurotrophins and Rho inhibitors upon neurite extension in vitro, we analyzed mice deficient in the *p75^{NTR}* gene for defects in nerve outgrowth during embryogenesis. The strain of mouse used carries a targeted deletion in the third exon of *p75^{NTR}*, which encodes the neurotrophin-binding domain of $p75^{NTR}$ (Lee et al., 1992). While a splice variant of $p75^{NTR}$ is still expressed in these mice, this truncated protein is unable to bind neurotrophins (Dechant and Barde, 1997). However, it is identical to the full-length form of $p75^{NTR}$ in its transmembrane and cytoplasmic domains (D. von Schack, G. Dechant, and Y.-A. B, unpublished data). Whole embryos between 10.0 and 12.5 days post coitum (dpc) were stained with the monoclonal antibody TuJ1, raised against the neuron-specific marker β tubulin III (Moody et al., 1989). *p75^{NTR}* −/− and

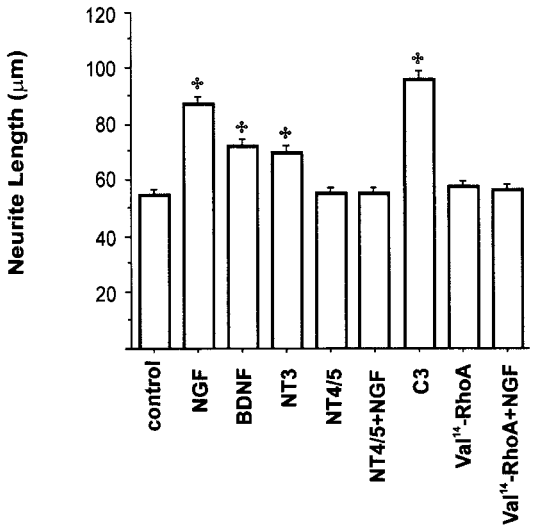

Figure 6. Ciliary Neurons: Quantification of Neurite Outgrowth

Neurotrophins were used at 50 ng/ml, except for NT-4/5 (50 μg/ml), and average neurite outgrowth per neuron was determined using cells fixed after 5 hr. The Rho inhibitory transferase C3 as well as the constitutively active RhoA mutant Val^{14}-RhoA were introduced by trituration into the cells before plating. The results represent means ± SEM from three to six separate experiments. The asterisks indicate values significantly different ($p < 0.05$ by paired t test) from control.

p75^{NTR} +/− littermates were compared, and intralitter differences in development were minimized by comparing embryos with equal somite number. At dpc 11.0 (42 somites), outgrowth of thoracic intercostal nerves in *p75*^{NTR} −/− embryos was clearly retarded compared to *p75*^{NTR} +/− littermates (Figure 7A). Analysis of dpc 12.0 embryos (49 somites) indicated a similar delay, though the intercostal nerves in *p75*^{NTR} −/− embryos did prove capable of ramification (Figure 7B). A significant delay was also observed in the innervation of superficial motor axons in the forelimb (Figure 7B). These defects were observed in all *p75*^{NTR} −/− embryos observed (n = 16, 4 litters). Comparison of wild-type embryos in separate litters, but with identical somite numbers, revealed nerve growth rates identical to that of *p75*^{NTR} +/− embryos (data not shown).

Discussion

A yeast two-hybrid screen has identified RhoA as a protein that interacts with the neurotrophin p75^{NTR} receptor. In vitro experiments established that p75^{NTR} acts as a constitutive Rho activator, that Rho activation is modulated by binding of neurotrophins to p75^{NTR}, and that this interaction affects neurite outgrowth from ciliary ganglion neurons. Examination of mice carrying a mutation in the *p75*^{NTR} gene revealed that the outgrowth of sensory and spinal axons during embryogenesis is delayed, compared with wild-type embryos.

Activation of Rho by p75^{NTR}

The Rho subgroup of Ras superfamily GTP-binding proteins is best known for its ability to control actin filament assembly and the formation and maintenance of focal adhesions (Ridley and Hall, 1992; Hotchin and Hall, 1996). The activity of these proteins is regulated by a variety of intracellular proteins. The guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, thereby activating their targets. The Rho GEFs consist of two families that differ in the structure of their catalytic domains. The first group comprises enzymes with a Dbl homology domain (reviewed by Mackay and Hall, 1998). The second subset is composed of Rho GDP dissociation stimulators (GDS; Boguski and McCormick, 1993). While the structure of the intracellular domain of p75^{NTR} does not immediately suggest that it contains a Dbl or GDS domain, it has been noted early on that a short sequence does show similarities with the 14-mer peptide mastoparan (Feinstein and Larhammar, 1990). Mastoparan is an amphiphilic component of wasp venom known to activate a number of GTP-binding proteins (Higashijima and Ross, 1991), including Rho (Koch et al., 1991). The mastoparan-like domain of p75^{NTR} is

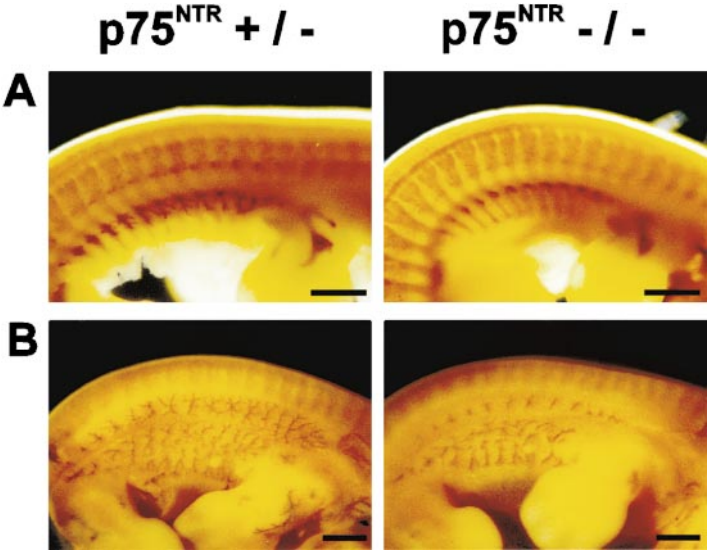

Figure 7. Reduced Outgrowth of Sensory and Motor Axons in *p75*^{NTR}-Deficient Mice

Whole-mount immunostainings of embryos at dpc 11.0 (A) and 12.0 (B) using the TuJ1 antibody, which recognizes the neuronal-specific protein β tubulin III. *p75*^{NTR} +/− and *p75*^{NTR} −/− littermates of the same somite number were compared. Note the reduced outgrowth in *p75*^{NTR} −/− mutants of intercostal nerves in the thoracic area and spinal nerves in the forelimb. Rostral is to the right, and dorsal is at the top of all panels. Scale bar, 0.5 mm.

now known to correspond to the fifth of the six α helices of p75^{NTR} (Liepinsh et al., 1997). In this context, it is interesting to note that the results of our experiments with subdomains of p75^{NTR} indicate that the Rho-interacting domain maps to the six α helix subdomain of p75^{NTR} (Figure 1).

Experiments with bacterially expressed p75^{NTR} ICD failed to indicate an GDP/GTP exchange activity on recombinant RhoA in in vitro assays. However, this may not be too surprising, as isoprenylation of Rho has been reported to be prerequisite for its activation by mastoparan (Koch et al., 1992). Posttranslational modifications of p75^{NTR} that may take place in yeast or 293 cells could also be required for p75^{NTR} to exert its activity on Rho.

If p75^{NTR} would function as a simple transmembrane GEF for Rho, it would be expected that the expression of p75^{NTR} into Swiss 3T3 fibroblasts may lead to the formation of stress fibers. Somewhat unexpectedly, this turned out not to be the case (C. D. Nobes and A. Hall, personal communication). It is possible that the expression of endogenous inhibitors of Rho in Swiss 3T3 cells, such as the Rho-GDP dissociation inhibitor GDI, may be sufficient to prevent the activation of Rho by a membrane-bound Rho activator like p75^{NTR}.

p75^{NTR} and Other Signaling Molecules

While Rho appears to be the GTPase reported to associate with p75^{NTR}, there is rapidly growing evidence for the association of this receptor with other molecules. In particular, p75^{NTR} has been shown to associate with the three other known neurotrophin receptors, TrkA, TrkB, and TrkC (Bibel et al., 1999), as well as with TRAF6 (Khursigara et al., 1999) and a zinc finger–containing molecule designated NRIF (for neurotrophin receptor interacting factor; Casademunt et al., 1999). The diversity of these associations is not surprising given the number of biological properties reported for p75^{NTR}. The association with Trk receptors explains increases in ligand affinity and specificity (Mahadeo et al., 1994); the recruitment of TRAF6 likely relates to the ability of p75^{NTR} to activate NF-κB (Carter et al., 1996); and NRIF appears to be involved in cell killing mediated by p75^{NTR} (Casademunt et al., 1999). How the activation of Rho fits with these previously described activities of p75^{NTR} remains to be seen. Rho has previously been reported to activate NF-κB (Mackay and Hall, 1998) and is discussed in the context of programmed cell death (reviewed by Gomez et al., 1998). However, both in Schwann cells and in the developing retina, the p75^{NTR}-mediated activation of NF-κB and of cell death have been shown to be ligand dependent (Carter et al., 1996; Frade et al., 1996), suggesting that the relationships with the constitutive activation of Rho reported here, if any, may not be straightforward.

p75^{NTR} and Rho in the Nervous System

Our observations indicating a link between p75^{NTR} and Rho, and thus to the actin cytoskeleton, are especially intriguing in view of the developmental pattern of expression of p75^{NTR}. It has long been observed that this receptor is expressed early in neurogenesis, during the initial phase of axonal elongation, as well as on neural crest cells. Ligand binding to p75^{NTR} would lead to decreased levels of Rho activation, and this could modulate the formation of actin filaments in such a way that the rigidity of the actin cytoskleton is decreased. The mechanisms by which Rho affects neuronal morphology will need to be studied in detail, as our results open the possibility that spatially restricted exposure of p75-bearing cell bodies to neurotrophins may favor localized axonal or dendritic outgrowth. In our assay with ciliary neurons, we observed that while all three neurotrophins tested enhance the rate of fiber outgrowth, NGF seems to be more effective than BDNF or NT-3 (Figure 6). Of note is that in the [^{32}P]GDP-Rho immunoprecipitation assay, NGF is also more effective than either BDNF or NT-3 in its downregulation of Rho activity (Figure 3B). With regard to experiments with cultured neurons, recent observations by others indicate that blocking Rho activation increases the rate of neurite outgrowth (Jin and Strittmatter, 1997; reviewed by Gallo and Letourneau, 1998). These neurons also express p75^{NTR}, which presumably caused the activation of Rho proteins in sensory neurons.

The reason why axonal elongation is retarded in mutant animals may be related to the lost ability of these animals to bind neurotrophins in a p75^{NTR}-dependent fashion. This would be in line with the results obtained with ciliary neurons (Figure 6). Interestingly, there is evidence for the presence of a second transcript encoding a protein lacking the neurotrophin-binding domain, but with an intact cytoplasmic domain (D. von Schack, G. Dechant, and Y.-A. B, unpublished data; see also Dechant and Barde, 1997). It is conceivable that the levels of this truncated form of p75^{NTR} contribute to significant, ligand-independent levels of Rho activation. In future studies, it will be interesting to determine the levels of Rho activity in growing neurites in this p75^{NTR} mutant, compared with wild-type embryos. Finally, we note that axonal elongation is retarded but that it does not stall. Analyses at later time points are complicated by the fact that there is a substantial decrease in the number of sensory neurons in this p75^{NTR} mutant (Stucky and Koltzenburg, 1997). In contrast, no cell loss is observed in sympathetic ganglia in this mutant (Brennan et al., 1999). Interestingly, a previous study noted that in sympathetic structures such as the pineal and subgroups of sweat glands, innervation by p75^{NTR}-bearing sympathetic terminals is absent in the p75^{NTR} mutant, even in the adult (Lee et al., 1994).

With regard to cell migration, a previous study indicated that treatment with NGF caused an increase in migration of Schwann cells, which, like most neural crest derivatives, express high levels of p75^{NTR} (Anton et al., 1994). We note that neural crest cells express not only p75^{NTR} (see, e.g., Fariñas et al., 1996) but also RhoB and RhoC (Liu and Jessell, 1998). As may be expected in view of their high degree of structural relatedness, p75^{NTR} activates not only RhoA but also RhoB. The possibility exists then that the expression of p75^{NTR} by neural crest cells and their derivatives is used as a means to modulate their migratory behavior.

In conclusion, the finding of the constitutive activation of Rho by p75^{NTR} reveals an aspect of the biology of this receptor that is in line with its expression pattern. It suggests a means by which axonal growth cones, neural

crest cells, and their derivatives may rapidly reorganize their actin cytoskeleton as a result of binding ligands encountered during their navigation.

Experimental Procedures

Yeast Two-Hybrid Analysis

A yeast GAL4 two-hybrid screen was performed essentially as described (Vojtek et al., 1993; see also Stratagene library protocol). Chick p75^{NTR} ICD, helical domain (HD), or juxtamembrane linker region cDNAs were prepared by polymerase chain reaction and ligated into the vector pBDGAL4-Cam. The *Saccharomyces cerevisiae* strain HF7c was transformed with one of these vectors and the vector PAD-GAL4-2.1 fused with a library of cDNA fragments from chick embryonic (E5) neural retina (oligo (dT)–primed Stratagene custom two-hybrid cDNA library made from poly(A)$^+$ RNA). Approximately 8×10^6 transformants were screened, and 40 clones were isolated from the His-autotrophic and lacZ-positive colonies. Verification of specificity of interaction between the baits and target proteins were then performed by cotransformation, and finally two true positive clones were obtained.

Coimmunoprecipitation of p75^{NTR} and RhoA

Using the calcium phosphate precipitation method, 293 cells were transfected with pcDNA3 vectors containing HA-RhoA wt, or HA-Val^{14}-RhoA (provided by Y. Takai), or chick p75^{NTR} ICD. After 48 hr, the cells were lysed on ice for 20 min with lysis buffer (20 mM Tris–HCl [pH 7.5], 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 0.05% NP40, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). The lysates were centrifuged at 13,000 × g for 20 min, and the supernatant was collected. They were then incubated with an anti-HA antibody (Boehringer Mannheim) for 2 hr, followed by incubation with protein Sepharose A (Pharmacia) for 1.5 hr at 4°C. The suspension was centrifuged at 1,000 × g for 5 min. The pellets were washed four times with lysis buffer and subjected to SDS–PAGE followed by immunoblot analysis using polyclonal anti-human p75 antibody (Promega).

In Vivo Nucleotide Labeling

RhoA, RhoB, and RhoC were N-terminally HA tagged and cloned into pcDNA3 expression vectors. Chick p75 ICD and full-length chick p75 constructs in pcDNA3 were also used. 293 cells or HN10 cells were transfected using the calcium phosphate method. After transfection, cells were cultured for 48 hr, serum starved in phosphate-free DMEM medium, labeled with [^{32}P] orthophosphate (100 μCi/ml) for 16 hr, and disrupted in lysis buffer (50 mM Tris–HCl [pH 7.5], 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet-P40, 1 mM sodium orthovanadate, 1 mM PMSF, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). Lysates were immunoprecipitated with an anti-HA monoclonal antibody (Boehringer) for 2 hr using protein Sepharose A beads (Pharmacia/LKB). Immunoprecipitates were washed three times in lysis buffer and twice in washing buffer (50 mM Tris–HCl [pH 7.5], 20 mM $MgCl_2$, and 500 mM NaCl) and finally were resuspended in 1 M KH_2PO_4 (pH 3.4). Bound nucleotides were released by heating at 68°C and fractionated using polyethyleneimine thin-layer chromatography plates. Radioactive spots, located by autoradiography, were scraped off the plates and counted in a scintillation β counter. While the [^{32}P] levels were low both in control and in neurotrophin-exposed samples, they are comparable with those determined by others using this method (Laudanna et al., 1996).

Affinity Precipitation of GTP-Rho

Transfected 293 cells were lysed in 50 mM Tris (pH 7.5) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, and 10 μg/ml each of leupeptin and aprotinin. Cell lysates were clarified by centrifugation at 13,000 × g at 4°C for 10 min, and the supernatants were incubated with the 20 μg of GST-Rho-binding domain of Rhotekin beads at 4°C for 45 min (Ren et al., 1999). The beads were washed four times with washing buffer (50 mM Tris [pH 7.5] containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, and 10 μg/ml each of leupeptin and aprotinin). Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

RT–PCR

Poly(A)$^+$ RNA from E10 and E14 chick ciliary and from E10 dorsal root ganglia were extracted using the micro mRNA purification kit (Pharmacia), followed by cDNA synthesis using SuperScript Reverse RNase-transcriptase (Gibco BRL). TrkA primers corresponded to amino acids 1443–1462 and 1186–1205 (Schröpel et al., 1995), glyceraldehyde-3-phosphate dehydrogenase primers corresponded to amino acids 659–678 and 1186–1205, and chick p75^{NTR} primers comprised 5′-CCTGCCTGGACAGTGTGACC-3′ and 5′-TCTGCC AGGGTGGTGGCC-3′ (Large et al., 1989). p75^{NTR} and TrkA cDNAs were amplified for 33 cycles (59°C for 30 s, 72°C for 1 min, 94°C for 30 s) and GAPDH cDNA for 30 cycles (65°C for 30 s, 72°C for 1 min, 94°C for 30 s).

Recombinant Proteins

Val^{14}-RhoA protein and *C. botulinum* C3 transferase were produced in bacteria as glutathione S-transferase (GST) fusion proteins, according to the manufacturers' instructions (Pharmacia), and then treated with Precision protease (Pharmacia) or thrombin (Pharmacia), respectively, to remove the GST moiety (Nobes and Hall, 1995). Thrombin was removed from the samples by aspiration to minobenzamidin-agarose.

Chick Ciliary Ganglion Neurons and Neurite Outgrowth

Ciliary ganglia were removed from stage 35 White Leghorn chicken embryos and dissociated into single cells by using trypsin as described (Collins and Dawson, 1983). For trituration experiments, neurons were suspended in 25 mM Tris–HCl, 150 mM NaCl, and 5 mM $MgCl_2$ [pH 7.5], with Val^{14}-RhoA at 5 mg/ml or with C3 transferase at 0.1 mg/ml. They were triturated by slowly passing the suspension through a narrow bore pipette (Borasio et al., 1989; Jin and Strittmatter, 1997). The final concentration of NGF, BDNF, and NT-3 used was 50 ng/ml. The efficiency of the trituration procedure for neurons was monitored by trituration with FITC-dextran (Sigma), and more than 95% of neurons were found to be strongly fluorescent. After trituration, neurons were plated in 25 vol of culture medium consisting of Ham's F12 medium (Gibco) containing 10% fetal calf serum. Plastic culture dishes were coated sequentially with 100 μg/ml poly-L-ornithine (Sigma) and 20 μg/ml laminin (Sigma). For outgrowth assays, plated cells were incubated for 5 hr and were fixed in 2% (w/v) glutaralaldehyde, and the neurite length per neuron was measured (Collins and Dawson, 1983).

Immunocytochemistry

Cultured neurons were fixed for 20 min at room temperature with 4% paraformaldehyde containing 0.1% glutaraldehyde in PBS [pH 7.4] and 120 mM sucrose and were washed three times with PBS. Cells were permeabilized with 0.5% Triton X-100/PBS and washed sequentially three times with PBS. Cultures were incubated overnight with monoclonal antibodies either to chick p75^{NTR} (provided by H. Tanaka) or to RhoA (Santa Cruz), followed by a Cy5-labeled anti-mouse monoclonal antibody for 1 hr. The specificity of the antibodies was assessed by Western blot analysis of cells expressing the proteins, and control experiments of immunocytochemistry were performed by leaving out the primary antibodies.

Whole-Mount Staining

The strain of mice bearing a targeted disruption of the third exon of the *p75^{NTR}* gene (Lee et al., 1992) was originally obtained from the Jackson Laboratory (Bar Harbor, Maine) on a mixed 129/SvJae and BALB/c background. A wild-type control colony was derived from the +/+ progeny of mated *p75^{NTR}* +/− siblings. *p75^{NTR}* −/− males were paired overnight with *p75^{NTR}* +/− females, and females displaying vaginal plugs were separated in the morning. Noon of this day is indicated as 0.5 dpc. Embryos were removed individually in cold PBS, somite number was counted as a gauge to developmental stage, the head was sliced open dorsally to allow antibody penetration, and a small piece of the tail was removed to produce DNA for genotyping. PCR genotyping proceeded as described (Frade and Barde, 1999). Eviscerated embryos were processed separately in a whole-mount staining procedure using a monoclonal antibody (TuJ1) recognizing the neuron-specific β tubulin III protein (Moody et al., 1989), essentially as described (Lee et al., 1995).

All incubations were carried out on a rocking table and at room temperature, except for fixation and antibody incubations, which were done at 4°C. Embryos were placed in fixative (methanol:DMSO 4:1) overnight and then bleached with fixative:30% H_2O_2 (5:1) for 6 hr. Embryos were rehydrated to PBS/0.25% Triton X-100 by successive 1 hr incubations in 80%, 50%, 30%, and 0% methanol, followed by a 2 day incubation in blocking solution (fetal calf serum [Gibco BRL]:DMSO 4:1, with 0.1% thimerosal) with a 1:200 dilution of TuJ1 (Berkeley Antibody Company). Embryos were washed six times for 1 hr each in PBS and then incubated overnight in blocking solution containing a 1:200 dilution of horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody (PIERCE). Embryos were then washed as before and incubated for 1 hr in 0.3 mg/ml diaminobenzidine and 5 mg/ml $NiCl_2$. H_2O_2 was added to 0.03% and the development reaction stopped after 10–30 min by a wash in PBS. Embryos were cleared in glycerol and photographed.

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